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Ionophore and Anthelmintic Activity of PF 1022A, a Cyclooctadepsipeptide, Are Not Related†

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Abstract: PF 1022A, a 24-membered cyclooctadepsipeptide and a potent anthelmintic drug, is active against nematodes but not against arthropods. Muscle cells of *Ascaris suum* generate autorhythmic spikes in electrophysiological control experiments. Exposure of the worm to PF 1022A leads to flaccid paralysis and in parallel to the disappearance of these spike events. Results of such experiments *in vitro* were compared with those from experiments using planar lipid bilayer membranes incorporating PF 1022A, a related linear octadepsipeptide and other cyclodepsipeptides.

Whereas PF 1022A acts both as an ionophore in lipid bilayers, similar to other cyclodepsipeptides like valinomycin and enniatin A, and as a paralysing drug in worms, some of the series of depsipeptides examined have only an ion carrier function, while others exhibit only nematocidal activity. It is concluded that the ion carrier property of PF 1022A is not responsible for its paralyzing effect on nematodes and that there is a specific binding site for PF 1022A in nematodes. Binding may trigger the lethal reaction cascade, which is responsible for anthelmintic activity.

Key words: anthelmintic activity, *Ascaris suum*, carrier, cyclodepsipeptide, ionophore, lipid bilayers, nematodes, PF 1022A

1 INTRODUCTION

The cyclooctadepsipeptide PF 1022A was recently isolated from the fungus imperfectus *Mycelia sterilia* and structurally characterized by Sasaki *et al.*[1] It is the most active member of a novel class of anthelmintic drugs[2] and several routes for its synthesis have been reported.[3–6] PF 1022A is active against *Angiostrongylus cantonensis* Mackerras and Sanders,[7] *Haemonchus contortus* Rudolphi[8] and other nematodes. Tests *in vitro* on the following nematodes and arthropods indicated good anthelmintic activity with nematodes but no activity against arthropods: *Trichinella spiralis* Owen larvae, adult *Nippostrongylus brasiliensis* Laue worms, adult *Heterakis spumosa* Schneider worms, adult *Boophilus microplus* Canestrini ticks and *Aedes aegypti* L. mosquito larvae and pupae.

In order to investigate the paralyzing action of PF 1022A on nematodes the neuromuscular system of *Ascaris suum* Goeze, which is assumed to behave similarly to *Ascaris lumbricoides* L., was chosen for membrane potential measurements.[9,10] Piperazine produces a flaccid paralysis of *A. lumbricoides*,[11] so the influence of PF 1022A on the resting potential and on the spontaneous occurrence of spike-like potential changes of somatic muscle cells was investigated for comparison.

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Cyclodepsipeptides such as valinomycin, enniatin A and beauvericin are potent ionophores which have been structurally characterized in organic solvents[12] and their ion carrier properties studied in great detail in planar lipid bilayer membranes.[13–16] PF 1022A was tested for ionophore activity in such a lipid model system in order to find an explanation for its action on nematodes.

To check whether nematode paralysis is caused by the ionophore activity of PF 1022A or by another mechanism, a number of structurally related and unrelated cyclodepsipeptides was studied as follows: the optical antipode PF 1022-001 (in which the D and L conformations are juxtaposed with respect to PF 1022A); the linearized cyclooctadepsipeptide SJB 1822; SJB 1878, a chlorinated cyclotetradepsipeptide having half the size of PF 1022A; the cyclohexadepsipeptides enniatin A_1, beauvericin and JES 1798, 2315 and 2103, three newly synthesized enniatins.

In this paper we present experimental evidence that the ion carrier function of PF 1022A is not the cause of nematode paralysis.

2 MATERIALS AND METHODS

2.1 Synthesis of PF 1022A and enniatin derivatives

PF 1022A was synthesized as described in detail elsewhere.[6] The macrocyclic optical antipode, PF 1022-001, was synthesized by the same procedure but using the appropriate D- and L-isomer starting components (see Table 1). The sample of PF 1022-001 used in our investigations was kindly provided by Dr Iinuma, Meiji Seika Kaisha, Ltd, Japan. The structures of compounds used in the investigations are given in Table 1.

The *N*- and *C*-protected octadepsipeptide SJB 1822, consisting of two identical tetradepsipeptide units, is a precursor of the corresponding (L-MePhe)PF 1022A in the synthesis pathway before stepwise deprotection and macrocyclization.[6]

SJB 1878 is a chlorophenyl-lactic acid-containing tetradepsipeptide (Table 1). Macrocyclization had been carried out to produce a half-size compound for anthelmintic activity testing.

The enniatins A, A_1, B and B_1 were purchased from Paesel & Lorei, Frankfurt, Germany, beauvericin from Sigma, Deisenhofen, Germany and valinomycin from Fluka, Neu-Ulm, Germany. The structure of enniatin-type depsipeptides was modified synthetically in a way similar to that for PF 1022A and its derivatives.[17]

2.2 Tests *in vitro*

T. spiralis larvae were isolated from skeletal muscles and diaphragm of male SPF/CFW1 mice and stored in aqueous sodium chloride (9 g litre^{-1}), supplemented with canesten (20 μg ml^{-1}). Larvae (20 per test) were

TABLE 1
Structure of Depsipeptides used in This Work[a]

Cyclododecadepsipeptide
Valinomycin: cyclo(-D-HyIv-D-Val-L-Lac-L-Val-)$_3$

Cyclooctadepsipeptides
PF 1022A: cyclo(-D-Lac-L-MeLeu-D-PhLac-L-MeLeu-)$_2$
PF 1022-001: cyclo(-L-Lac-D-MeLeu-L-PhLac-D-MeLeu-)$_2$ (optical antipode)

Linear octadepsipeptide
SJB 1822: Bn-(-L-MeLeu-D-Lac-L-MeLeu-D-PhLac-)$_2$-O-*t*Bu

Cyclohexadepsipeptides
Enniatin A: cyclo(-D-HyIv-L-MeIle-D-HyIv-L-MeIle-D-HyIv-L-MeIle-)
Enniatin A_1: cyclo(-D-HyIv-L-MeIle-D-HyIv-L-MeIle-D-HyIv-L-MeVal-)
Enniatin B: cyclo(-D-HyIv-L-MeVal-D-HyIv-L-MeVal-D-HyIv-L-MeVal-)
Enniatin B_1: cyclo(-D-HyIv-L-MeVal-D-HyIv-L-MeVal-D-HyIv-L-MeIle-)
Beauvericin: cyclo(-D-HyIv-L-MePhe-D-HyIv-L-MePhe-D-HyIv-L-MePhe-)
JES 1798: cyclo(-D-Lac-L-MeAla-D-Lac-L-MeIle-D-Lac-L-MeIle-)
JES 2315: cyclo(-Hyac-L-MeAla-D-Lac-L-MeIle-D-Lac-L-MeIle-)
JES 2103: cyclo(-D-Lac-L-MeAla-D-Lac-L-MeAla-D-Lac-L-MeAla-)

Cyclotetradepsipeptide
SJB 1878: cyclo(-L-MeLeu-D-Lac-L-MeLeu-D-4-Cl-PhLac-)

[a] Bn = benzyl; *t*Bu = *tert*-butyl; L-MeAla = *N*-methyl-L-alanine; L-MeIle = *N*-methyl-L-isoleucine; L- or D-MeLeu = *N*-methyl-L-leucine or -D-leucine; L-MePhe = *N*-methyl-L-phenylalanine; L-MeVal = *N*-methyl-L-valine; L- or D-Val = L- or D-valine; Hyac = 2-hydroxyacetic acid; D-HyIv = D-2-hydroxy*iso*-valeric acid; L- or D-Lac = L- or D-lactic acid; L- or D-PhLac = L- or D-phenyllactic acid; D-4-Cl-PhLac = D-(4-chlorophenyl)lactic acid.

incubated in a solution (2 ml) with the following composition (g litre^{-1}): Bacto casitone (20), yeast extract (10), glucose (5), KH_2PO_4 (0·8), K_2HPO_4 (0·8); pH 7·2; supplemented with sisomycin and canesten (10 and 1 μg ml^{-1}, respectively). The test compound (10 mg) was dissolved in dimethylsulfoxide (DMSO; 0·5 ml) and added to the incubation medium to give a final concentration of 100, 10 or 1 μg ml^{-1}. The mixtures were incubated for five days at 19°C and anthelmintic activity was then ranked on a scale 0–3 where 3 = full activity (all larvae were dead); 2 = good activity (many dead larvae were present); 1 = weak activity (number of living larvae was less than control); 0 = no activity (number of living larvae was equal to that in the control).[18]

Adult *N. brasiliensis* worms were isolated from the small intestine of female Wistar rats and stored in aqueous sodium chloride (9 g litre^{-1}) supplemented with sisomycin and canesten (20 and 2 μg ml^{-1}, respectively). Five worms (mixed sexes) were incubated in that medium (1·0 ml), which was used for estimation of acetylcholine esterase (AChE) activity.[19] Test depsipeptides were added in the same way as for *T. spiralis* larvae tests and anthelmintic activity was ranked after incubation for six days at 37°C using the following scale: 3 = full activity (complete AChE inhibition); 2 = good activity (>75% enzyme inhibition); 1 = weak activity (50–75% AChE inhibition) and 0 = no activity (<50% enzyme inhibition).

Adult *H. spumosa* worms were isolated from the upper colon of male SPF/CFW1 mice and stored in a salt medium containing (g litre^{-1}): NaCl (8), KCl (0·175), $CaCl_2$ (0·175), $MgCl_2$ (0·1) and glucose (5 mM), K_2HPO_4 + KH_2PO_4 (1·25 mM); pH 7·2, supplemented with ampicillin, streptomycin and canesten (50, 50 and 10 μg ml^{-1}, respectively). Female worms (10) were incubated in this medium (1·5 ml) for three days at 37°C, test compounds being added in the same way as for *T. spiralis* larvae tests. Anthelmintic activity was ranked on the scale 0–3, where 3 = full activity (total inhibition of egg release and paralysis of all worms); 2 = good activity (paralysis of more than seven worms or >75% inhibition of egg release); 1 = weak activity (5–7 worms paralyzed or 50–75% inhibition of egg release) and 0 = no activity (fewer than five worms paralyzed or <50% inhibition of egg release).

Adult, fully-engorged female *B. microplus* ticks were injected with a DMSO solution (1 μl) containing the appropriate depsipeptide concentration (usually between 0·2 and 20 μg per tick). Activity was assessed in terms of inhibition of egg-laying or reduction of the numbers and weight of eggs using the scale 0–3, where 0 represents no inhibition and 1–3 represent total, 50–99% and <50% inhibition, respectively.

Larvae/pupae from eggs of the mosquito *A. aegypti*, which had been kept in plastic dishes at 23°C for up to 21 days to complete their development and fed daily on 'Tetramin'^{R} liquid, were treated with the test compound, which was added to their food. The test compounds in acetone (2 g litre^{-1}) were diluted and added to the food to give a final concentration of 0·01–10 mg ml^{-1}. Activity was determined as described for the *T. spiralis* assay.

2.3 *Ascaris suum* preparation and electrophysiological measurements

A. suum worms were collected at the local slaughterhouse and kept at 37°C in Ascaris–Ringer solution (NaCl 130; KCl 24; $CaCl_2$ 6; $MgCl_2$ 5; MES 5 mM; pH 6·7) which was changed daily. Experiments started at least one day and worms were discarded two or three days after collection.

For tests of antiparasitic activity *in vitro*, three worms per experiment were incubated for up to 24 h in Ascaris–Ringer solution (250 ml) supplemented with PF 1022A or one of the other depsipeptides. Test compounds were dissolved in DMSO to yield stock solutions of 20 mg ml^{-1} and added to the incubation medium to a final concentration of 7·5 μg ml^{-1}. In control experiments, a comparable amount of DMSO (4·2 mM) was added to the incubation medium.

Paralyzing effects were investigated over a 24-h period at increasing time intervals starting at 30 min. Overall motility of the worms was judged by eye as well as upon mechanical stimulation.

For membrane potential measurements of somatic muscle cells from *A. suum*, a nerve-muscle preparation was obtained according to the procedure of Baldwin and Moyle.[9] Female worms were pinned, ventral side down, to a wax-coated preparation dish under coverage of Ringer solution. The dorsal muscle mass was divided longitudinally, deflected and pinned down. Then the gut was removed with fine forceps, exposing the muscle cell bodies. A section of 2–3 cm length directly anterior to the genital pore was excised and fixed in the experimental chamber cuticle side down.

Membrane potentials were measured using two microelectrodes. One was placed in the bag or bulge region of muscle cells and the second in the bath solution. Microelectrodes were pulled from aluminium silicate capillary glass filled with 2 M KCl and had resistances of 1–2 MΩ. The differential amplifier (Turbo Tec 01C) was from NPI Electronic, Frankfurt, Germany. Signal was filtered at 1·5 kHz using a Bessel-type filter (PABF 10N, NPI Electronic), digitized (ITC 16 interface, Instrutech Corp., Elmont, NY, USA) and recorded on a Power MacIntosh computer (software PULSE from HEKA, Lambrecht, Germany). Data were analyzed on an IBM-compatible PC using in-house programs (G. Geßner, unpublished).

Resting potentials and potential spike activity of muscle cell membranes were usually recorded from the

nuclear bag or bulge region.[10] Since some preparations showed a short-lasting lag phase with respect to the appearance of spike activity, measurements were started after a waiting time of 15 min. At least eight successive recordings, each of 20 s length, were gathered. From each worm at least 10 randomly selected cells were measured. If spike activity could not be detected, 10–40 further cells were tested to confirm the result. All experiments were carried out at 37°C in Ascaris–Ringer solution.

2.4 Experiments in planar lipid bilayers

Lipid bilayer membranes were formed from a solution of 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine (1,2-POPC) and 1,2-dioleoyl-glycero-3-phosphatidyl-ethanolamine (1,2-DOPE) (80 + 20 by mass) in hexane (4 mg ml^{-1}). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification.

Depsipeptides were dissolved in ethanol, typically at a concentration of 4 mg ml^{-1}. Varying amounts of this stock solution were added to the lipid/hexane solution and this mixture was used to form the bilayer membranes. Experiments were carried out in 0·5 M and 0·25 M alkali chloride (Li^+, Na^+, K^+, Rb^+ or Cs^+) solutions at room temperature (21°C).

Set-up chambers were made from Teflon. Furthermore, a Teflon foil (6 μm thick) was fixed between the two parts of the chamber. Into this septum, a hole (typically 0·2 mm diameter) was punched by application of an electric spark. The bilayer was formed onto that hole by apposition of two monolayers according to the method of Montal and Mueller.[20,21]

The sign of membrane voltage refers to the *cis*-compartment with respect to the grounded *trans*-compartment. Current was measured with a home-made current-to-voltage converter providing a transimpedance of 1 mV pA^{-1}. Recordings were stored on a DAT recorder (DTR 1200, Science Products, Hofheim, Germany). For data evaluation on an IBM-compatible PC, self-written programs were used.[22]

3 RESULTS

3.1 In-vitro studies of depsipeptide action on nematodes and arthropods

3.1.1 In-vitro tests with nematodes

Results of tests *in vitro* for anthelmintic activity of the depsipeptides are listed in Table 2, from which it is clear that the enniatins A, A_1, B and B_1 generally possess the best anthelmintic activity *in vitro* against *T. spiralis*, while that against the other two nematodes is lower. Beauvericin, another 18-membered cyclodepsipeptide and valinomycin, a 36-membered cyclodepsipeptide, exert only a weak anthelmintic activity *in vitro*. While PF 1022A, a 24-membered cyclodepsipeptide, exerts very good anthelmintic activity at 1 μg ml^{-1} against all three nematodes tested, its antipode PF 1022-001 showed only minor activity against *H. spumosa*. This suggests that a specific binding site for PF 1022A is responsible for its high anthelmintic activity and this was demonstrated very recently by Pleiss *et al.*[23]

3.1.2 In-vitro tests with arthropods

Data for activities against the arthropods *B. microplus* and *A. aegypti* larvae are also listed in Table 2. With

TABLE 2
Anthelmintic Activity *in vitro* against Various Nematodes and Arthropods

Depsipeptides	N. brasiliensis *Activity*[a]	T. spiralis *Activity*[a]	H. spumosa *Activity*[a]	B. microplus		A. aegypti	
				Dose[b]	*Activity*[a]	*Dose*[c]	*Activity*[a]
Valinomycin	3	2	n.d.[d]	0·2	3	10	3
PF 1022A	2	3	3	20	3	>10	0
PF 1022-001	1	1	1	>20	0	>10	0
SJB 1822	0	0	n.d.	>20	0	>10	0
Enniatin A	2	3	n.d.	>20	0	>10	0
Enniatin A_1	0	3	2	>20	0	>10	0
Enniatin B	1	2	0	>20	0	>10	0
Enniatin B_1	1	2	2	>20	0	>10	0
Beauvericin	2	1	0	>20	0	>10	0
SJB 1878	3	3	n.d.	>20	0	>10	0

[a] On a scale 0 (no activity) to 3 (full activity); for details see text.
[b] μg injected per fully engorged tick.
[c] μg ml^{-1}.
[d] n.d., not determined.

the exception of valinomycin, which was fully active against *B. microplus* at 0·2 $\mu g\ ml^{-1}$ injected per female tick, all cyclohexadepsipeptides displayed weak or no activity against the two arthropods. In presence of PF 1022A a significant effect was observed only at the high dose of 20 μg per tick. In contrast, no activity at all was seen with the antipode PF 1022-001 and with the linearized depsipeptide SJB 1822.

3.2 Studies of depsipeptide action on *Ascaris suum*

3.2.1 In-vitro paralysis tests with Ascaris suum

Experiments *in vitro* with *A. suum* worms clearly demonstrated the paralysing effect of PF 1022A. Spontaneous motility disappeared after *c.* 2 h incubation in Ascaris–Ringer supplemented with 7·5 $\mu g\ ml^{-1}$ PF 1022A, although a slight movement could be seen upon mechanical stimulation. After 5 h the worm showed the flaccid paralysis which is typical of worms treated with piperazine.[11] The linear depsipeptide SJB 1822, applied at the same concentration, induced the same paralyzing effect, but approximately twice the time was needed for complete flaccid paralysis as compared to PF 1022A. In contrast, the optical antipode PF 1022-001 showed no significant influence on the motility of *A. suum* within 14–20 h.

Reduction of PF 1022A concentration led to a slower paralysis reaction. Change of the bath medium to unsupplemented Ascaris–Ringer and overnight incubation did not cause recovery of the worm from paralysis. The PF 1022A effect is thus irreversible. Control experiments involving addition of DMSO without the test substance (4·2 mM) to the incubation medium showed no effect within 24 h in these experiments.

3.2.2 Electrophysiological measurements with Ascaris suum *cells*

In order to investigate the electrophysiological basis of muscle paralysis, the membrane potential of single *A. suum* muscle cells was measured. The worm was incubated in Ascaris–Ringer supplemented with DMSO (4·2 mM) alone, or a solution of PF 1022A, SJB 1822 or PF 1022-001 (7·5 $\mu g\ ml^{-1}$, Figs 1A–D). Then intracellular recordings were made after 5 and 14 h from muscle cells and the time-course of membrane potential change recorded.

Due to the insolubility in water of the depsipeptides studied, DMSO was used as solvent for a stock solution. Since DMSO diffuses into a membrane after addition to the surrounding solution and exerts detergent-like effects, we carried out control experiments with DMSO. If both Ringer solutions, the incubation medium of the worm and the bathing medium of the muscle cells were supplemented with DMSO, characteristic irregular potential spikes were observed after

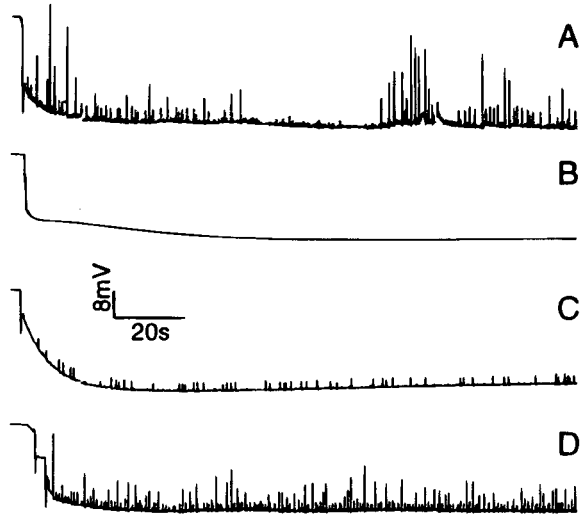

Fig. 1. Effect of PF 1022A, SJB 1822 and PF 1022-001 on the spontaneous irregular potential spikes in *Ascaris* suum muscle cells. Downward deflection at the left of the traces (A–D) indicates the point of muscle cell impalement. A. Control experiment with DMSO (4·2 mM) in Ascaris–Ringer worm incubation medium and muscle cell bathing medium during membrane potential recording. After 5 h of worm incubation and subsequent preparation of muscle cells, spontaneous irregular potential spikes still appear in the same way as in DMSO-free test experiments. B. The presence of PF 1022A (7·5 $\mu g\ ml^{-1}$) in the incubation medium leads to a complete loss of spike activity within 5 h. C. As with PF 1022A, the linear octadepsipeptide SJB 1822 (7·5 $\mu g\ ml^{-1}$) causes a strong reduction in the number of potential spikes after 5 h. After 14 h, activity was completely abolished. D. In contrast to PF 1022A and SJB 1822, the optical antipode PF 1022-001 induces no activity change in bursting behaviour of potential spikes even after 14 h of incubation.

5 h, similar to those in experiments without any DMSO ($n = 107(\pm 44)\ min^{-1}$ (number of spikes >0·1 mV); Fig. 1A). However, after 14 h of incubation spike events had almost completely vanished. On the other hand, if the bath medium was kept free of DMSO, no change in the bursting of potential spikes was observed within 24 h.

Figure 1B clearly shows that after 5 h of incubation with PF 1022A the muscle cells were quiescent. After perfusion with a PF 1022A/DMSO-free Ascaris–Ringer solution, potential spikes did not reappear, i.e. cells did not recover. It is concluded that the PF 1022A effect is irreversible also on the cell membrane level.

The octadepsipeptide SJB 1822 is still potent in paralyzing the worm and suppresses the potential spikes of muscle cells, but with lower efficiency. In Fig. 1C it can be seen that after 5 h of incubation potential spikes occurred at reduced frequency ($n = 67(\pm 47)\ min^{-1}$), but after 14 h spike events had completely disappeared (data not shown).

In contrast to PF 1022A and SJB 1822, the optical antipode PF 1022-001 has no effect on spike activity within 14 h of incubation ($n = 111(\pm 38)\ min^{-1}$; Fig. 1D) and even longer (though its stock solution solvent,

DMSO, is present). Consequently, it behaves completely differently from PF 1022A and the linearized depsipeptide.

3.3 Planar lipid bilayer experiments

3.3.1 Ion carrier properties of PF 1022A

Since PF 1022A has a good solubility in ethanol, this cyclodepsipeptide was added as an ethanolic stock solution (4 mg ml^{-1}) to the electrolyte solution of one of the set-up compartments in the same way as is usually done with valinomycin and the enniatins.[13–15] Due to the very low solubility of PF 1022A in water, we observed only a slight increase in membrane conductivity within 30–60 min and, in addition, wide scattering of the data.

Because of this insufficient experimental reproducibility, we added 50–150 µl of the ethanolic stock solution to 500 µl bilayer-forming solution. First we started with *n*-decane-containing lipid bilayers, which were formed according to the original technique of Mueller and Rudin.[22,24,25] Immediately after thinning to a black membrane, bilayer conductivity was quite high, but then it decreased gradually to low values over 10–20 min. This is probably a consequence of drug separation into the *n*-decane/lipid torus which surrounds the bilayer.

Alternatively, we used the virtually solvent-free lipid bilayer system developed by Montal and Mueller[20,21] and added 100 µl of PF 1022A stock solution to 500 µl lipid/hexane membrane-forming solution. After spreading this mixture on the electrolyte surface and volatilisation of the hexane solvent, a planar bilayer was formed by apposition of the two lipid monolayers. The induced conductivity was virtually constant during a time period of 10–30 min and was reproducible within a factor of 2–3. Consequently we chose the latter lipid system for our studies.

The current/voltage (I/V)-characteristic of a lipid bilayer modified by PF 1022A (Fig. 2) is best described by a sinh function as follows

$$I = k \sinh(\alpha V), \quad \text{with } \alpha = \delta \frac{F}{RT},$$

where F, R, T are the Faraday constant, gas constant and absolute temperature, respectively.

In Fig. 2 this is shown for all alkali chlorides tested. The sinh-dependency favours a model for ion transport, in which the ion has to pass a single energy barrier[14] caused by the inner hydrocarbon core of the lipid bilayer. In the case of a symmetric barrier one theoretically expects $\delta = 0{\cdot}5$ for this parameter, which quantifies the voltage dependence of I. At low membrane voltages $\sinh(\alpha \cdot V)$ is approximated by the linear function $\alpha \cdot V$. Therefore we are able to calculate a conductivity value λ from

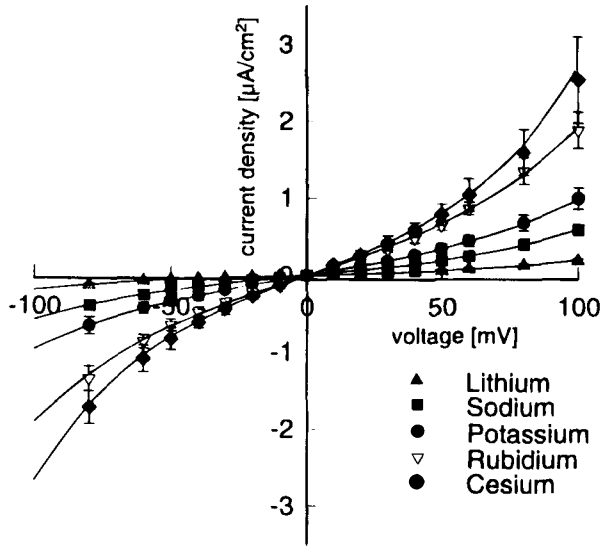

Fig. 2. Ion carrier property and alkali ion selectivity of PF 1022A in planar lipid bilayer membranes. The I,V-curves drawn show a sinh-functional dependence of carrier-induced currents on membrane voltage. The selectivity sequence $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ corresponds to Eisenman's sequence I. For further details see text. Experimental conditions: Virtually solvent-free planar bilayers were formed from 1,2-POPC + 1,2-DOPE (80 + 20). PF 1022A was added to the membrane-forming solution to yield a molar test compound : lipid ratio of *c.* 1 : 5. All alkali chloride solutions were 0·5 M, buffered at pH 7·2; temperature 15°C.

$$I = \lambda V, \quad \text{with } \lambda = k\alpha.$$

The corresponding parameter values for the five alkali chlorides (0·5 M salt solutions) are listed in Table 3.

In addition to λ and δ, the selectivity ratios on the basis of conductivities with respect to the most permeant Cs^+-ion are also listed in Table 3. The selectivity sequence $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ corresponds to Eisenman's selectivity sequence I.

3.3.2 Ion carrier properties of enniatin A_1 and derivative

Figure 3 shows the comparison of bilayer-modifying properties of the cyclodepsipeptides PF 1022A, PF

TABLE 3

Conductivities λ, Selectivity Ratios $\lambda(\text{Ion})/\lambda(Cs^+)$ and Voltage Dependence Indicating Parameter δ for Alkali Chloride Solutions[a]

Ion	$\lambda(\mu S\ cm^{-2})$	$\lambda(Ion)/\lambda(Cs^+)$	δ
Lithium	1·1	0·08	0·50
Sodium	3·4	0·26	0·48
Potassium	6·2	0·47	0·43
Rubidium	11·8	0·89	0·45
Cesium	13·3	1·00	0·55

[a] For experimental details see Fig. 2 and text.

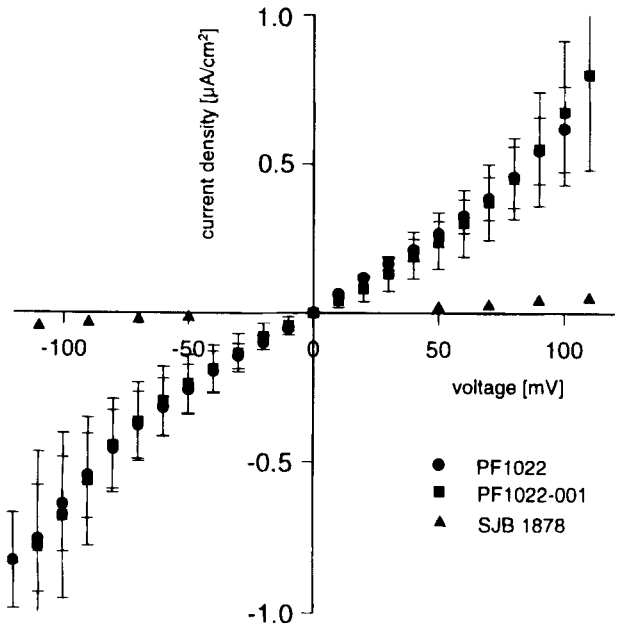

Fig. 3. Comparison of bilayer modifying properties of the cyclodepsipeptides PF 1022A, PF 1022-001 and SJB 1878. For details see text. Experimental conditions: Molar test compound : lipid ratio 1 : 5 for PF 1022A, PF 1022-001 and 2 : 5 for 1878. Salt 0·25 M KCl, temperature 21°C. Otherwise same as in Fig. 2.

1022-001 and SJB 1878. It is clear that the cyclooctadepsipeptide PF 1022A and its optical antipode PF 1022-001 induce nearly the same high bilayer conductivity while, in contrast, the half-size cyclo-

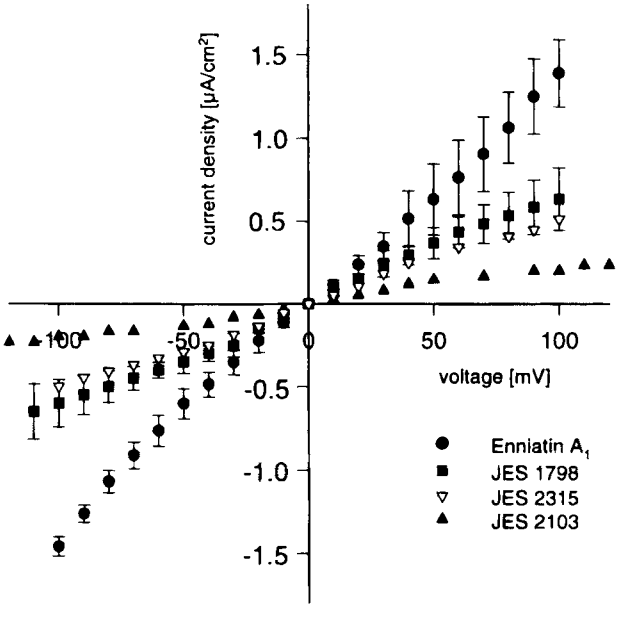

Fig. 4. Comparison of bilayer-modifying properties of the cyclohexadepsipeptides enniatin A_1 and the synthetic enniatins JES 1798, JES 2315 and JES 2103. For details see text. Experimental conditions: Molar test compound : lipid ratio 1 : 10 for enniatin A_1 and 1 : 3 for JES 1798, JES 2315 and JES 2103. Salt 0·25 M KCl, temperature 21°C. Otherwise same as in Fig. 2.

tetradepsipeptide SJB 1878 exhibits only a small current increase above the bare membrane level.

It should be noted that the linear octadepsipeptide SJB 1822 is completely inactive. *I/V*-curves in the presence of SJB 1822 are virtually identical to those of the unmodified lipid bilayer (data not shown).

The comparison of the bilayer-modifying properties of the cyclohexadepsipeptides enniatin A_1 and the synthetic derivatives JES 1798, JES 2315 and JES 2103 can be seen in Fig. 4. Except for enniatin A_1, which induces a somewhat higher bilayer conductivity, all three synthetic enniatins give rise to a conductivity increase of the same order of magnitude as that with PF 1022A (Fig. 3).

4 DISCUSSION

In order to study the mode of action of PF 1022A, a number of depsipeptides were investigated using the three approaches of activity *in vitro* against nematodes and arthropods, activity against *A. suum* worms and studies using planar bilayers. The results are summarized in Table 4.

Of the materials tested, PF 1022A proved to be the most potent in killing nematodes but was virtually ineffective against arthropods. Although less potent, the cyclododecadepsipeptide valinomycin, the cyclohexadepsipeptides enniatin A_1 and JES 1798 and the cyclotetradepsipeptide SJB 1878 showed good activity and even the linear octadepsipeptide SJB 1822 exhibited significant anthelmintic activity. On the other hand, the 18-membered cyclohexadepsipeptides beauvericin, JES 2315 and JES 2103 were only weakly active against nematodes. It is important to note that the optical antipode of PF 1022A, PF 1022-001, which has the opposite D- and L-isomeric pattern, had virtually no effect on nematodes and arthropods. Since the ion-complexing properties of PF 1022-001 should be very similar to those of PF 1022A, the steric orientation of the side chains of these depsipeptides seems to be important for the anthelmintic activity. The presence of a quite specific binding site at a postulated protein molecule for a particular segment of the PF 1022A sequence would explain this fact. The preserved activity found with the linear octadepsipeptide SJB 1822 is in good agreement with this concept.

Two methods were used with *A. suum* worms, paralysis tests *in vitro* with incubated worms and membrane potential measurements of muscle cell preparations. The two methods gave similar results. PF 1022A caused worm paralysis within 4–5 h at the concentration used; the linear compound SJB 1822 was slightly less active but, in contrast, PF 1022-001 caused no paralysis within 24 h.

In electrophysiological potential measurements, muscle cells exhibit autorhythmic spikes of several mV

TABLE 4
Comparison of Ion Carrier Function in Planar Lipid Bilayers, Paralyzing Effect on *Ascaris suum* and Anthelmintic Activity against Nematodes

	Planar bilayers[a]			
Depsipeptides	λ ($\mu S\ cm^{-2}$)	C ($mg\ ml^{-1}$)	Ascaris suum[b]	Nematodes[b]
Valinomycin	28·9	0·00067	n.d.[c]	+
PF 1022A	4·9	0·67	++	++
PF 1022-001	4·3	0·67	−	−
SJB 1822	<0·1	0·67	+	+
Enniatin A_1	11·9	0·23	n.d.	+
Beauvericin	11·1	0·15	n.d.	−
JES 1798	7·5	0·67	n.d.	+
JES 2315	6·3	0·67	n.d.	−
JES 2103	2·5	0·67	n.d.	−
SJB 1878	0·3	0·67	n.d.	+

[a] Bilayer conductivity λ and test compound concentration C in lipid-hexane solution; conductivity of an unmodified planar lipid bilayer was typically 0·1 $\mu S\ cm^{-2}$.
[b] ++ (highly active), + (active), − (no activity) (from Fig. 1 and Table 2, respectively).
[c] n.d., not determined.

amplitude, which usually appear in irregular bursts. Muscle cells of worms in flaccid paralysis caused by exposure to PF 1022A or SJB 1822 did not generate any spiking of the membrane potential. Incubation of paralyzed worms or cell preparations in drug-free Ringer solution did not lead to a recovery of the potential spike activity. In contrast, PF 1022-001 had virtually no effect on the occurrence of the potential spikes, even after incubation for 14 h.

Consistent with our experiments on nematodes, the studies on *A. suum* preparations clearly show that cation complexation cannot be the explanation for the anthelmintic activity of PF 1022A as the antipodean compound PF 1022-001 is inactive. The flaccid appearance of the paralyzed worm, as well as the irreversibility of paralysis, have to be considered when seeking an explanation of the killing process, which may have been due to exhaustion of metabolic or energy resources. In addition, the insolubility of PF 1022A in aqueous solutions, and thus insufficient diffusion from the possible binding site, or very strong binding of PF 1022A to this site could be the cause for irreversibility of paralysis.

The third series of experiments involved the planar lipid bilayer technique for a study of the ionophore, i.e. ion carrier, properties of the depsipeptides tested. Table 4 indicates that, in comparison to valinomycin, the ability to modify the bilayer conductivity is relatively low for all the smaller cyclodepsipeptides. In general, the performances of the cyclooctadepsipeptides and the cyclohexadepsipeptides as ionophores are comparable. The cyclotetradepsipeptide SJB 1878 seems to be too small for effective cation complexing and leads to only a negligible increase in bilayer conductivity. The linear octadepsipeptide SJB 1822 is completely inactive. Folding of the molecule around a cation seems to be energetically unfavourable; instead it may be adsorbed onto the membrane interface with its hydrophobic side chains orientated towards the bilayer and with its carbonyl oxygens towards the aqueous phase.

Although results of bilayer experiments confirm the expectation that PF 1022A and its optical antipode PF 1022-001 do not differ in their activities as ionophores, they have very different anthelmintic activities. On the other hand, the linear octadepsipeptide SJB 1822 and the small cyclotetradepsipeptide SJB 1878 do not act as ion carriers, but they are potent in killing nematodes. Similar arguments apply to the enniatins and the synthetic cyclohexadepsipeptides. Consequently we conclude that the ion carrier properties of PF 1022A are not responsible for its anthelmintic activity.

Further studies on nematodes and corresponding cell preparations will have to be carried out in order to identify the postulated high-affinity binding site for PF 1022A, although such a site was identified very recently in *A. suum*.[23]

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